Soil Biology and Biochemistry 118 (2018) 130–144

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Responses of bulk and rhizosphere soil microbial communities to thermoclimatic changes in a Mediterranean ecosystem

Javier Pascual[1], Silvia Blanco, Juan Luis Ramos, Pieter van Dillewijn*

Department of Environmental Protection, Estación Experimental Del Zaidín, C.S.I.C., Calle Profesor Albareda 1, 18008 Granada, Spain

ARTICLE INFO

Keywords:
Altitudinal gradient
Microbial diversity
Rhizosphere
Thymus zygis
Thermoclimate
Climate change

ABSTRACT

The effect of thermoclimatic changes on microbial communities in the rhizosphere of a wild thyme species, *Thymus zygis* L., and its surrounding bulk soil was studied along elevational gradients in the Sierra Nevada National Park (Spain). Multiplex amplicon sequencing of bacterial and fungal taxonomic markers revealed that the richness, diversity and structure of bacterial and fungal communities were affected by thermoclimatic changes, with environmental parameters (mean annual atmospheric temperature and precipitation) and edaphic properties (mainly pH and nutrients) as the major drivers. Although both bulk soil and rhizosphere communities were structured according to the thermoclimatic zones, the response of microorganisms to thermoclimatic changes was different depending on the rhizosphere effect. On the contrary, the microbial functional gene diversity was not affected by thermoclimatic changes suggesting functional redundancy in the microbial communities along the altitudinal gradients. However, the functional gene diversity was clearly different between bulk soil and the rhizosphere, with the latter harbouring a larger number of gene copies and more different functional genes than bulk soils. Finally, a set of microbial bioindicators are defined for the thermoclimatic zones as a starting point to develop improved biological tools and models to monitor and predict the effects of climate changes. To the best of our knowledge, this is the first study where the response of bulk soil and rhizosphere microbial communities to thermoclimatic changes has been studied in parallel.

1. Introduction

Microorganisms, including bacteria and fungi, exist in complex and dynamic communities in the soil where they form the basis for terrestrial ecosystems and biogeochemical cycles. The rhizosphere, which is the soil under the influence of plant roots (Hartmann et al., 2008), is particularly important for plant health and nutrition (Lakshmanan et al., 2014) and also plays roles in biogeochemical cycles (Philippot et al., 2008) and soil formation (Séguin et al., 2005; Drigo et al., 2008). In this biotope, the flow of nutrients released by plants as root exudates (Bais et al., 2006) affect microbial activity, richness and community composition so that microbial assemblages differ from those found in soil further away from the root, the so-called bulk soil. The microbial communities in the rhizosphere are influenced by the species and the physiological state of the plant but also by soil characteristics (Bulgarelli et al., 2013; Philippot et al., 2013; Chaparro et al., 2014; Lebeis, 2015; Llado et al., 2017). However, the effect of other environmental factors such as climate remains largely unknown, thereby limiting the accuracy of models formulated to predict and mitigate the possible adverse effects of climate change (Gärdenäs et al., 2011).

To determine the effect of climate change on microbial communities, altitudinal gradients in the mountain ranges that span multiple thermoclimatic zones in a short geographic distance are receiving much attention in recent years. As such, several studies have focused on taxonomic and functional changes in soil microbial communities along these gradients (Bryant et al., 2008; Fierer et al., 2011; Lin et al., 2015; Yasir et al., 2015; Lanzen et al., 2016; Siles et al., 2016; Siles and Margesin, 2017; among others). With the exception of Fierer and collaborators (2011), most of these studies have shown community changes at different altitudes although different tendencies were reported regarding the relationship between bacterial diversity and increasing elevation such as no trends (Yasir et al., 2015; Lanzen et al., 2016), decreasing trends (Bryant et al., 2008; Shen et al., 2013, 2015; Wang et al., 2015a; Wang et al., 2015b; Zhang et al., 2015), increasing trends (Siles and Margesin, 2016) hump-backed trends (Singh et al., 2012; Lin et al., 2015) or trends with a dip at lower mid elevations

* Corresponding author.
E-mail addresses: javier.pascual@dsmz.de (J. Pascual), silvia.blanco@eez.csic.es (S. Blanco), juanluis.ramos@eez.csic.es (J.L. Ramos), pieter.vandillewijn@eez.csic.es (P. van Dillewijn).
[1] Present address: Department of Microbial Ecology and Diversity Research, Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

https://doi.org/10.1016/j.soilbio.2017.12.013
Received 16 October 2017; Received in revised form 12 December 2017; Accepted 15 December 2017
Available online 26 December 2017
0038-0717/

Table 1
Soil physicochemical properties and the estimated environmental factors. MAT, Mean Atmospheric Annual Temperature; MAP Mean Annual Precipitation.

Region	Thermoclimatic zone (Mediterranean)	Altitude (m)	pH	Total nitrogen (%)	Carbonates (%)	Oxidizable organic material (%)	Assimilable phosphorus (p.p.m.)	Assimilable potassium (p.p.m.)	MAT (ºC)	MAP (mm)
Capileira	Meso-	1100	7.08	0.1	1.2	1.5	3	65	17.2	494
	Supra-	1400	6.77	0.06	2.5	0.9	1	80	16.2	564
	Supra-	1700	5.86	0.13	1.6	2.1	3	75	14.8	639
	Oro-	2000	6.45	0.08	1.6	1.2	3	130	12.8	680
	Oro-	2300	5.72	0.48	1.5	6.4	5	180	12.0	728
Puerto de la Ragua	Meso-	1100	7.91	0.05	24	0.7	0	19	15.3	451
	Supra-	1400	6.36	0.12	1.6	1.9	2	89	14.9	472
	Supra-	1700	6.31	0.12	1.8	2	2	93	12.8	550
	Oro-	2000	6.41	0.14	1.6	1.7	3	59	11.5	603
	Oro-	2300	6.15	0.16	1.8	1.6	6	35	10.3	643

(Singh et al., 2014). With regard to fungal diversity along elevational gradients, these generally follow the same trends as those observed with bacterial diversity at the same location but not always (Wang et al., 2015a; Siles and Margesin, 2016). A possible explanation for the contradicting microbial diversity trends found in these studies could be due to the influence of local abiotic soil properties and biological factors which cannot easily be separated from those caused by climatic factors (Lanzen et al., 2016). As a result, obtaining additional data from other mountain systems could be of interest to carry out comparative studies (Hofmann et al., 2016). Moreover, more information of the functional gene diversity along altitudinal gradients may contribute to improve our understanding of how microbial communities respond to climate changes (Yang et al., 2014; Shen et al., 2016). As elevational gradient studies have so far been limited mostly to the microbial communities of bulk soil, expanding these studies to rhizosphere communities may give additional insights on the response of microbial communities harbored within this key ecological niche to climate change.

The Sierra Nevada National Park in southern Spain, is located in the Sierra Nevada mountain range within the Baetic System. The Park hosts predominantly high mountain shrublands and oak forests with flora which follow well described thermoclimatic zones found at different altitudes (Rivas-Martinez et al., 1997). These thermoclimatic zones are associated with the Mediterranean macrobioclimate consisting of high temperatures and low rainfall during the summer months and low temperatures during the winter. This Mediterranean biodiversity hotspot is a biosphere reserve integrated into national and international programs dedicated to monitoring the ecological impact of climate change (Zamora et al., 2015), which together with its marked stratification converts Sierra Nevada to be an ideal location for studies of subsoil microbial communities. Amongst the flora found in the Sierra Nevada, the wild thyme species, *Thymus zygis* L., is a cosmopolitan plant capable of growth at different altitudes and different soil types which along with its ecological importance in Mediterranean shrublands and its economic importance for the pharmaceutical, culinary and cosmetic industries (Pascual et al., 2016), makes it an interesting candidate for use as a model plant for rhizosphere studies.

In this study, the microbial communities in bulk soil and in the rhizosphere soil under the influence of *T. zygis* were analyzed across three thermoclimatic zones (Meso-, Supra- and Oro-Mediterranean), along two thermoclimatic transects in spatially distant regions in the Sierra Nevada Mountains during two consecutive years. The aim of this study was (i) to determine how the bacterial and fungal communities respond to thermoclimatic changes from a taxonomic and functional point of view and (ii) whether the responses are similar in bulk and rhizosphere soils. Furthermore, microbial bioindicators of the thermoclimatic zones were sought. We hypothesize that bulk and rhizosphere soil microbial communities are driven by deterministic factors associated with the thermoclimatic zonation. As a consequence of the selective ecological forces to which microorganisms are exposed at each thermoclimatic zone, we expect to find several bacterial and fungal species which could be used as potential bioindicators for future studies.

2. Material and methods

2.1. Sampling, processing and soil physicochemical characteristics

Soil and rhizosphere samples were taken in the spring of 2013 and 2014 along elevational gradients at two different regions within the Sierra Nevada National Park, Granada, Spain. Specifically, samples were collected at five different altitudes comprising three thermoclimatic zones (Meso-Mediterranean, 1107 ± 5.0 m; Supra-Mediterranean, 1402 ± 5.7 m, 1732 ± 27.3 m; and Oro-Mediterranean, 1998 ± 6.0 m, 2288 ± 33.2 m), on the south facing slopes of two regions within the Sierra Nevada Mountains; one near to the municipality of Capileira and another near to the mountain pass Puerto de la Ragua (Table S1). Each year, at each sampling area and at each altitude three adult and apparently healthy *Thymus zygis* L. plants of 10–15 cm height were collected within a maximum distance of 36 m from each other. Collected plants with intact roots and adhering soil were transported to the laboratory in sterile bags where the rhizosphere soil was separated from the roots as described in Pascual et al. (2016). At the same time as plants were collected, three soil samples were taken within approximately 1 m of each extracted plant by excavating to a depth of 10–15 cm. Soil samples were stored in sterile 50 ml Falcon tubes and transported to the laboratory. A total of 60 bulk and 60 rhizosphere soil samples were collected and further processed (3 bulk/rhizosphere soil replicates x 2 transects x 5 altitudes x 2 years).

Alongside the other soil samples, a large soil sample comprising of 1 dm^3 of thoroughly mixed bulk soil was taken at each sampling point during the first year and used for physicochemical characterization at the Andalusian soil analysis laboratory (Laboratorio Agroalimentario de Atarfe, Granada, Spain) using standard international methods. Each soil sample was characterized regarding soil pH, oxidizable organic matter, total nitrogen, assimilable phosphorous and potassium and carbonates. All collected soils have a sandy loam texture (Table 1). The physicochemical parameters of rhizosphere soil could not be measured due to the confounding effect of the buffer used to obtain this soil fraction as well as the limited quantities of rhizosphere soil obtained which were insufficient for proper analyses. Therefore, in this study, comparison of microbial diversity and composition with regard physicochemical edaphic parameters were limited only to bulk soils. Climatic factors MAT (Mean Atmospheric Annual Temperature) and MAP (Mean Annual Precipitation) were estimated for each sampling point using the tools available at https://cambia.climasig.es/# within the CNCM3 model and low emissions for the period 2011-2040 (Table 1).

2.2. DNA extraction, amplicon library construction of the 16S rRNA gene and intergenic region (ITS), and next generation sequencing

Metagenomic DNA was extracted from 0.5 g rhizosphere or bulk soil using the Fast DNA Spin Kit for Soil (MP Biomedicals LLC) following the manufacturer's instructions. A single metagenomic DNA extraction was carried out per soil sample. To study the bacterial communities, amplicon libraries were constructed from each sample by amplification by PCR of the V1-V3 region of the 16S rRNA gene using the eubacterial primers at *E. coli* position 8 (5′-AGAGTTTGATCMTGGCTCAG-3′) and position 532 (5′-TACCGCGGCKGCTGGC-3′) (Brosius et al., 1978) appended with the 454 A or B fusion sequence, respectively, together with a 4 bp key tag and 10-11 bp barcode. PCR reactions were performed with 0.3 ng of metagenomic DNA, 250 μM of each of the four dNTPs, 1.5 mM of $MgCl_2$, 200 nM of each primer, 2.5 U of Taq DNA polymerase (Roche), and the appropriate buffer supplied by the manufacturer. The amplification program used was as follows: 1 cycle of 5 min at 95 °C; 30-35 cycles of 45 s at 95 °C, 45 s at 43-55 °C, and 2 min at 72 °C; and, finally, 1 cycle of 10 min at 72 °C. Amplified products were further purified by separation on 1% agarose gels and extraction from excised bands using the QIAQUICK Gel Extraction Kit (Qiagen, Germany). DNA integrity was checked by agarose gel electrophoresis and quantified fluorometrically with QUBIT (Invitrogen) to obtain equimolar mixtures containing each amplicon library. EmPCR and bidirectional pyrosequencing was performed by Macrogen (Seoul, South Korea) using Roche GS FLX Titanium technology.

To determine the fungal community structure, the internal transcribed spacer 2 (ITS2) intergenic region between the 5S and 28S rRNA genes was targeted for PCR using the adapter linked primers 5′-GTGA ATCATCGAATCTTTGAA-3' (ITS86F) and 5′-TCCTCCGCTTATTGATA TGC-3' (ITSR2) (Op De Beeck et al., 2014). Amplification was performed with 0.3 ng metagenomic DNA, 200 μM of each dNTP, 500 nM of each primer, 0.6 U of Q5 High-Fidelity DNA polymerase (New England Biolabs), and the appropriate buffer supplied by the manufacturer. The amplification program for PCR consisted of 1 cycle of 30 s at 98 °C; 30-35 cycles of 10 s at 98 °C, 15 s at 56 °C, and 30 s at 72 °C; and a final cycle of 2 min at 72 °C. Each PCR reaction was sent to the Unidad de Genómica (Fundación Parque Científico de Madrid, Spain) where samples were appended with identification barcodes and sequenced 2 × 250 bp with Illumina MiSeq.

All sequence files were submitted to the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) and are accessible in Bio-Project PRJNA369661.

2.3. Functional GeoChip analysis

To carry out the functional analysis of the microbial communities, GeoChip (He et al., 2007) analysis was performed. One representative sample was chosen from the three bulk soil samples or the three rhizosphere soil samples obtained during 2013 from each of the five altitudes of the Capileira zone. These 10 samples were then processed by Glomics Inc. (USA) where metagenomic DNA was obtained from each sample using the freeze grind, SDS lysis method described by Zhou et al., (Zhou et al., 1996). 1000 ng of the DNA obtained from each sample was used for hybridization with the GeoChip 5.0 containing 180000 gene probes using a protocol similar to that described by (Liang et al., 2010) and (Azarbad et al., 2015). Briefly, 1 μg of DNA from each sample was labeled with Cy-3 using random primers and Klenow fragment. Then the labeled products were purified with QIAquick (Qiagen) and dried. For hybridization the labeled DNA was resuspended in a hybridization solution containing the common oligo reference standard (CORS) (Liang et al., 2010), loaded onto the GeoChip slide, and hybridized for 20-22 h at 67 °C. After hybridization, slides were washed and imaged (NimbleGen MS 200 microarray scanner) as a Multi-TIFF. The resulting data was extracted using the Agilent Feature Extraction program and then loaded onto the GeoChip data analysis pipeline (ieg.ou.edu/microarray/). Spots were scored as positive and retained if the signal-to-noise ratio was greater than 2, and the coefficient of variation (CV) of the background was less than 1.3. In addition, spots with signal intensities below 250 were discarded. Data normalization and quality filtering were performed as described previously with the aid of the CORS (Liang et al., 2010). The GeoChip 5.0 contains 26922 probes for carbon cycle genes, 797 for electron transfer, 4332 for metal homeogenesis, 6493 for nitrogen related genes, 11591 for organic remediation, 3260 for phosphorus related genes, 4032 for secondary metabolism, 26306 for stress, 4739 for sulfur, 21152 for virulence, 2857 for virus related genes and 10380 probes related to other genes.

2.4. Bioinformatics analysis of microbial communities

Sequences of 16S rRNA gene amplicons obtained by pyrosequencing were analyzed using QIIME (Caporaso et al., 2010b) version 1.9.1. Reads were filtered for quality (Phred 27) and size (350-600bp). Potential chimera sequences were detected using usearch61 (Edgar et al., 2011) and removed from the datasets. Samples with less than 5000 reads were removed due to their low coverage. Sequences of the remaining samples were clustered into operational taxonomic units (OTUs) at 97% of sequence identity with QIIME (Caporaso et al., 2010b) following an open reference OTU picking strategy using UCLUST (Edgar et al., 2011). Representative sequences of each OTU were aligned with the SILVA database (v123; Quast et al., 2013) using PyNAST (Caporaso et al., 2010a).

For ITS amplicon library analysis, the Illumina produced fastq readpair files were stitched and filtered using the QIIME pipeline (Caporaso et al., 2010b) version 1.9.1. Sequences were clustered into operational taxonomic units (OTUs) at 97% of sequence identity with QIIME (Caporaso et al., 2010b) following an open reference OTU picking strategy using UCLUST (Edgar et al., 2011). For taxonomic assignment the RDP Classifier tool with a confidence threshold of 0.5 (Wang et al., 2007) in combination with the UNITE database version 7.0 (release date 22-08-2016) (Kõljalg et al., 2013) was used.

For beta-diversity analyses of both bacterial and fungal communities, OTU's making up less than 0.005% of the reads in the total dataset were removed and normalized with the cumulative sum scaling (CSS) algorithm (Paulson et al., 2013).

2.5. Ecological and statistical analyses

Alpha diversity [Richness, Shannon diversity index (H′) and Simpson's Evenness (E)] and beta diversity based on Bray-Curtis distances of bacterial and fungal communities were analyzed using QIIME v1.9.1 (Caporaso et al., 2010b) and with the R packages RAM (version 1.2.1.3) and phyloseq (version 1.16.2). For these analyses OTUs defined at 3% of sequence dissimilarity were used. Boxplots and heatmaps were obtained with the R package ggplot2 (version 2.2.1) or with Microsoft Office. Rarefaction curves of observed OTUs were generated with QIIME [alpha_rarefaction.py; default conditions]. Non-metric multidimensional scaling (NMDS) and canonical correspondence analyses (CCA) were performed with the R package vegan (version 2.4–2); [vegan: ordinate (method = “NMDS″, distance = “bray"] and [vegan:CCA(); default conditions], respectively. For the CCA analyses, the correlation of the canonical axes with the explanatory matrix was determined with the general permutation test [vegan:permutest (); nperm = 999; default conditions]. The collinearity among explanatory variables was tested with the Variance Inflation Factor (VIF) analysis implemented in the R package usdm (version 1.1–15) [usdm:permutest (); nperm = 999; default conditions]. To compare bacterial and fungal community assemblages among the three thermoclimatic zones (Meso-, Supra- and Oro-Mediterranean) in the bulk soil and the rhizosphere, the non-parametric PERMANOVA test was used in vegan [vegan:adonis (); default conditions], followed by Bonferroni-correction for multiplicity

adjustment. The interaction among the variables, thermoclimatic zone and the type of soil (bulk or rhizosphere), was analyzed with two-way PERMANOVA tests with Past software (Version 3.15; https://folk.uio.no/ohammer/past/). To contrast the means of numerical values (alpha diversity parameters or relative abundances of microbial taxa) among groups, the R package multcomp (version 1.4–6) was used [multcomp:glht (stats:aov (values ~ groups, data), mcp (groups = "Tukey"), vcov = vcovHC)] (Herberich et al., 2010). Multiple pairwise comparisons were performed for the three thermoclimatic zones of both types of soils (bulk and rhizosphere) together. The Pearson correlation coefficient was used to study the lineal relationship between environmental parameters with altitude along the spatial transects with the R package psych (version 1.7.5) [psych:corr.test (method = "pearson")]. The Spearman's rank correlation coefficient was used to study the lineal relationship between the environmental parameters with microbial alpha diversity indices [psych:corr.test (method = "spearman")]. The significance of correlations was computed using a two-tailed *t*-test.

The core microbiome of bacterial and fungal communities was determined for the three thermoclimatic zones for bulk soil and the rhizosphere. It was defined as those OTUs present in 100% of the samples corresponding to each thermoclimatic zone for each soil type (bulk or rhizosphere). The bioindicators of thermoclimatic zones were designated as those OTUs which are part of the core microbiome of only one thermoclimatic zone and furthermore have relative abundances higher and significantly different than the other thermoclimatic zones according to the multcomp test ($p < .05$). The fidelity and specificity of the selected bioindicadors to a thermoclimatic zone was further corroborated with the Indicator species analysis (IndVal) (Dufrene and Legendre, 1997). The IndVal was analyzed with the R package labdsv (version 1.8–0) [labdsv:IndVal (); default conditions].

Statistical analyses of the results obtained with GeoChips were performed using tools available in the Microarray Data Manager (http://ieg.ou.edu/microarray) (He et al., 2007, 2010). The total normalized signal intensity detected for each gene is indicative of the gene copy number. The multivariable analysis of the normalized signal intensities of functional genes was plotted as a Detrended Correspondence Analysis (DCA). Significance among groups (type of soil (bulk or rhizosphere) and thermoclimatic zones) was tested with the Analysis of similarities (ANOSIM) test. The canonical correspondence analyses (CCA) was performed using the R package vegan (version 2.4–2) [vegan:CCA(); default conditions], respectively. The correlation of the canonical axes with the explanatory matrix was determined by the general permutation test [vegan:permutest (); nperm = 999; default conditions]. Alpha diversity parameters and relative abundance of the microbial functional genes between bulk and rhizosphere soil samples were tested in R with the *t*-test for equal means [stats:t.test].

3. Results

3.1. Edaphic and environmental characteristics along the elevational gradients

The physicochemical properties of representative bulk soil samples and the environmental characteristics at each of the sampling areas within the two elevational transects are given in Table 1. Mean atmospheric annual temperature (MAT) and mean annual precipitation (MAP) showed a strong and significant negative and positive correlation with altitude (Table 2), respectively. While along the transect in the Capileira region only the assimilable potassium correlated positively with altitude, in the Puerto de la Ragua region, assimilable phosphorus and total nitrogen increased significantly with altitude. Although pH did not show a significant correlation with elevation, a clear decrease is observed between 1100 m and higher altitudes (Table 1, Table 2).

Table 2
Pearson correlation coefficient (r) between environmental and edaphic soil properties and the altitude. Significant correlation are indicated with asterisks: *$p < .05$; **$p < .01$; ***$p < .001$. MAT, Mean Atmospheric Annual Temperature; MAP Mean Annual Precipitation.

	Capileira	Puerto de la Ragua
MAT	−0.99***	−0.99***
MAP	0.99***	0.99***
pH	−0.82	−0.75
Total nitrogen	0.70	0.91*
Carbonates	−0.09	−0.70
Oxidizable organic material	0.70	0.48
Assimilable phosphorus	0.67	0.93*
Assimilable potassium	0.91*	0.009

3.2. Bacterial community composition

Amplicon products of the V1-V3 region of the 16S rRNA gene were obtained from each of the 120 samples and sequenced by 454 GS FLX pyrosequencing. A total of 3,066,594 sequences were obtained. Amplicon length and quality filtering followed by elimination of possible chimera sequences resulted in a total of 1,365,077 sequences with a mean length of 441 bp. In order to ensure sufficient coverage depth, samples with less than 5000 reads were eliminated from the study resulting in 92 samples with a total of 1,036,936 sequences. This reduction in the number of samples did not compromise any of the datasets because at least two out of the three replicates of each soil sample were retained for subsequent analyses. According to the rarefaction curves (Fig. S1A), the depth of sequencing of these samples was sufficient to cover the full diversity.

Analyses of the edaphic bacterial communities by nonmetric multidimensional scaling (NMDS) indicate that the main deterministic factors which affect the bacterial community structure are the type of soil (bulk or rhizosphere) and the thermoclimatic zone in which the communities are located within the altitudinal gradient (Fig. 1A). Neither the sampling year (2013 or 2014) or the transect region (Capileira vs Puerto de la Ragua, approximately 30 km apart from each other) showed a strong effect on structuring the bacterial communities (Fig. S2A). The bacterial communities collected at 1400 and 1700 m (both Supra-) on the one hand, and at 2000 and 2300 m (both Oro-) on the other, grouped partially in the NMDS plot (Fig. S2A). This indicates that the bacterial assemblage depends mainly on the thermoclimatic zone rather than altitude *per se*. Since the main deterministic factors, thermoclimatic zone and soil type, showed a reciprocal interaction (two-way PERMANOVA test; $p < .001$) the effect of the thermoclimatic zone on the structure of the bacterial communities was studied independently for either bulk soil or rhizosphere soil. The Meso-thermoclimatic zone could be clearly differentiated from the other two zones independently of the type of soil analyzed (PERMANOVA tests; $p < .001$ for bulk soil; $p < .001$ for the rhizosphere; Fig. 1A). Bacterial communities inhabiting in the Oro- and Supra-Mediterranean zones overlapped partially in the NMDS plot (Fig. 1A) independently of the rhizosphere effect suggesting that the transition from the Supra-to Oro-thermoclimatic zones was not as evident as that from the Meso- to Supra-zones. Nevertheless, pairwise comparisons confirmed that all three bacterial communities (Meso-, Supra- and Oro-) were different among each other, either in the bulk soil or in the rhizosphere (pairwise comparison PERMANOVA tests; $p < .05$). Furthermore, the Bray-Curtis intragroup distances in the Supra-zone was larger than in the Meso- and Oro-zones suggesting that at intermediate thermoclimatic zones the bacterial communities are more heterogeneous both in the bulk soil and in the rhizosphere (Fig. S3A; multcomp test, $p < .001$).

Analysis of OTU richness, Shannon's diversity index (H′) and Simpson's Evenness (E) showed that the bacterial communities did not have the same alpha diversity features along the thermoclimatic transects when bulk soil and the rhizosphere were compared (Fig. 2A).

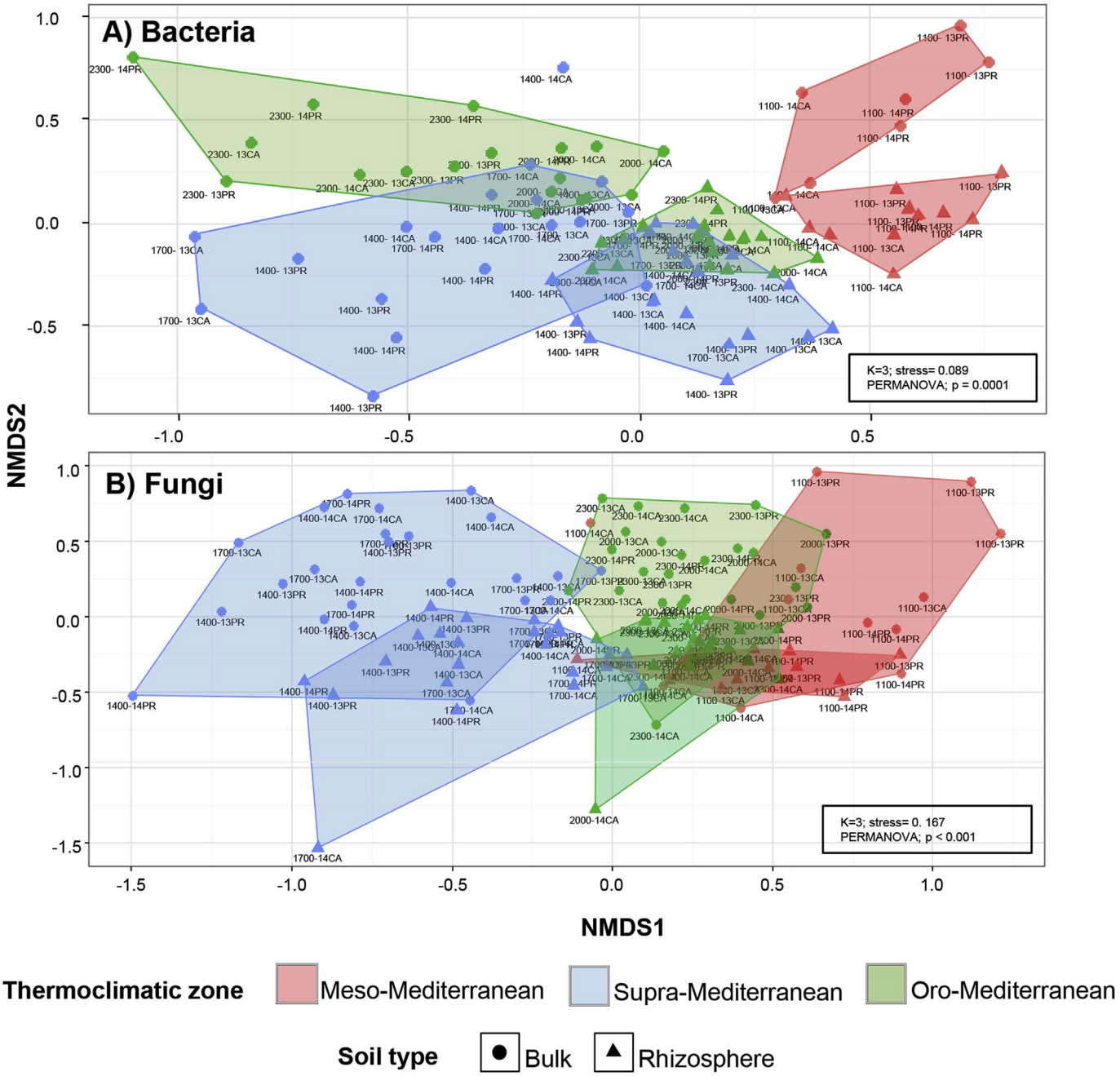

Fig. 1. Nonmetric multidimensional scaling (NMDS) based on Bray Curtis distances of A) bacterial and B) fungal communities. OTUs delimited at 3% dissimilarity. Sample 1400-CA from Bulk-Supra was excluded for the calculation of the convex hulls due to its outlier nature.

In bulk soil, the richness and diversity of OTUs did not show significant differences between the three thermoclimatic zones. However, the evenness decreased in the Supra- and Oro-Mediterranean zones, suggesting that a few numerically dominant OTUs inhabit these zones. In the case of the rhizosphere, the richness and diversity (H′) were reduced in the Supra-zone compared to the other two zones. The evenness of OTUs also tended to decrease although the differences were not significant among thermoclimatic zones. The richness of bulk soil bacteria correlated positively with MAP while the evenness of OTUs was correlated with different edaphic properties like pH (positively) and total nitrogen, organic matter and potassium (negatively) (Table 3). In the case of rhizosphere soil, a significant negative correlation between richness and MAT was evident.

Canonical correspondence analysis revealed that the environmental and edaphic chemical parameters affected the bulk soil bacterial communities differently along the thermoclimatic gradient (Fig. 3A). The proportion of total variability of bacterial communities attributed to the explanatory variables was 34%, and this partition of variability was significant (general permutation test, $p < .001$; 999 replicates; Fig. 3A). The pH and carbonate concentration, two directly interlinked parameters, showed a strong effect on bacterial assemblages in the Meso-Mediterranean zone particularly in the Puerto de la Ragua region. In the Meso-thermoclimatic zone, samples from either the Capileira or the Puerto de la Ragua regions could easily be differentiated from each other when the bacterial community was constrained with the explanatory factors, but not in the other two thermoclimatic zones. Independently of the geographical location of the transects, MAT was the main driver in the Supra-Mediterranean zone, while oxidizable organic matter, nitrogen, phosphorous, and to a lesser extent, potassium were the foremost environmental drivers in the Oro-Mediterranean zone.

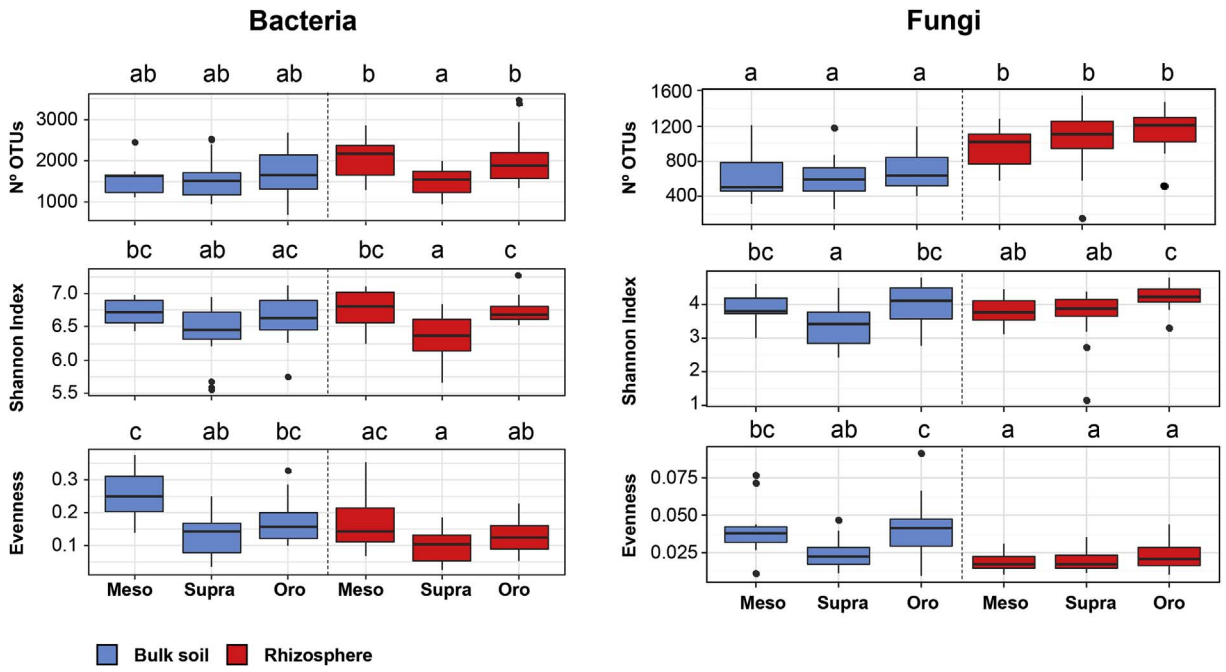

Fig. 2. Boxplots of alpha diversity indexes [Richness, Shannon's diversity index (H′) and Simpson's Evenness (E)] of A) bacterial and B) fungal communities based on OTUs defined at 3% sequence dissimilarity. Black dots represent outlier soil samples. Different letters indicate statistically significant differences according to multcomp tests ($p < .05\%$).

Table 3
Spearman's rank correlation coefficient between environmental and edaphic soil properties with alpha diversity indices of bacteria and fungi in bulk soils (3% OTUs dissimilarity). Significant correlations according to a two-tailed *t*-test are indicated with asterisks: *p < .05; **p < .01; ***p < .001. MAT, Mean Annual Temperature; MAP Mean Annual Precipitation; NA, data not available.

	Bacteria						Fungi					
	Bulk soil			Rhizosphere			Bulk soil			Rhizosphere		
	Richness	Shannon's index	Simpson's Evenness	Richness	Shannon's index	Simpson's Evenness	Richness	Shannon's index	Simpson's Evenness	Richness	Shannon's index	Simpson's Evenness
MAT	−0.06	−0.03	0.08	−0.09	−0.30*	0.16	0.18	0.21	0.20	−0.30*	−0.45***	−0.13
MAP	0.31*	0.11	−0.23	0.12	−0.26	−0.01	−0.12	−0.21	0.20	0.35**	0.47***	0.24
pH	−0.04	0.23	0.48**	NA	NA	NA	−0.04	0.21	0.23	NA	NA	NA
Total nitrogen	−0.01	−0.29	−0.44**	NA	NA	NA	0.08	0.05	0.08	NA	NA	NA
Carbonates	−0.25	−0.04	0.39	NA	NA	NA	−0.27*	−0.14	−0.03	NA	NA	NA
Oxidizable organic material	0.09	−0.24	−0.56***	NA	NA	NA	0.08	−0.19	−0.23	NA	NA	NA
Assimilable phosphorus	0.11	−0.08	−0.27	NA	NA	NA	0.16	0.30*	0.33*	NA	NA	NA
Assimilable potassium	0.26	0.08	−0.31*	NA	NA	NA	0.18	−0.20	−0.34**	NA	NA	NA

The taxonomic assembly at the phylum level of the bacterial communities along the three thermoclimatic zones was more variable in the bulk soil than in the rhizosphere. In both soil types, the edaphic bacterial communities harbored principally 16 different phyla, with *Actinobacteria* followed by *Proteobacteria* (mostly *Alphaproteobacteria*) and *Acidobacteria* as the most numerically dominant phyla (Fig. S4A). In either soil type, the abundance of *Chloroflexi* and *Planctomycetes* tended to increase as the thermoclimatic zone ascended. The most evident differences between bulk soil and rhizosphere soil bacterial communities were the larger abundances of *Acidobacteria*, *Gemmatimonadetes*, *Chloroflexi*, and candidate division AD3 in the bulk soil. On the other hand, *Proteobacteria,* mainly *Alphaproteobacteria* and *Gammaproteobacteria*, were more abundant in the rhizosphere.

At the family level, the most abundant taxa in both types of soils were *Bradyrhizobiaceae*, *Nocardioidaceae* and *Streptomycetaceae* (Fig. 4A). The strong niche partitioning observed for thermoclimatic zones is shown by the proliferation of specific bacterial families at each thermoclimatic zone. Independently of the rhizosphere effect on soil, the relative abundance of *Thermomonosporaceae* and EB1017 (an as yet not cultured family of the order *Acidimicrobiales*) increased as the thermoclimatic zone ascended. On the other hand, *Bacillaceae* decreased in the Supra-Mediterranean zone, while *Mycobacteriaceae* increased in this zone. For several families, the effect in the bulk soil and the rhizosphere at each thermoclimatic zone was different, with niche partitioning being stronger in the rhizosphere. In bulk soil, *Geodermatophilaceae*, *Solirubrobacteraceae* and *Sphingomonadaceae* diminished with descending thermoclimatic zone, while *Acetobacteraceae* increased. In the rhizosphere of wild thyme, *Gaiellaceae*, *Comamonadaceae*, *Acetobacteraceae* and *Oxalobacteraceae* showed larger relative abundances at higher thermoclimatic zones. Contrarily, the abundance of *Rhodospirillaceae*, *Micromonosporaceae* and *Sinobacteraceae* declined as the thermoclimatic zone descended. Whereas *Micrococcaceae* showed higher abundances in the Supra-Mediterranean zone *Propionibacteriaceae* and *Streptosporangiaceae* displayed lower abundances.

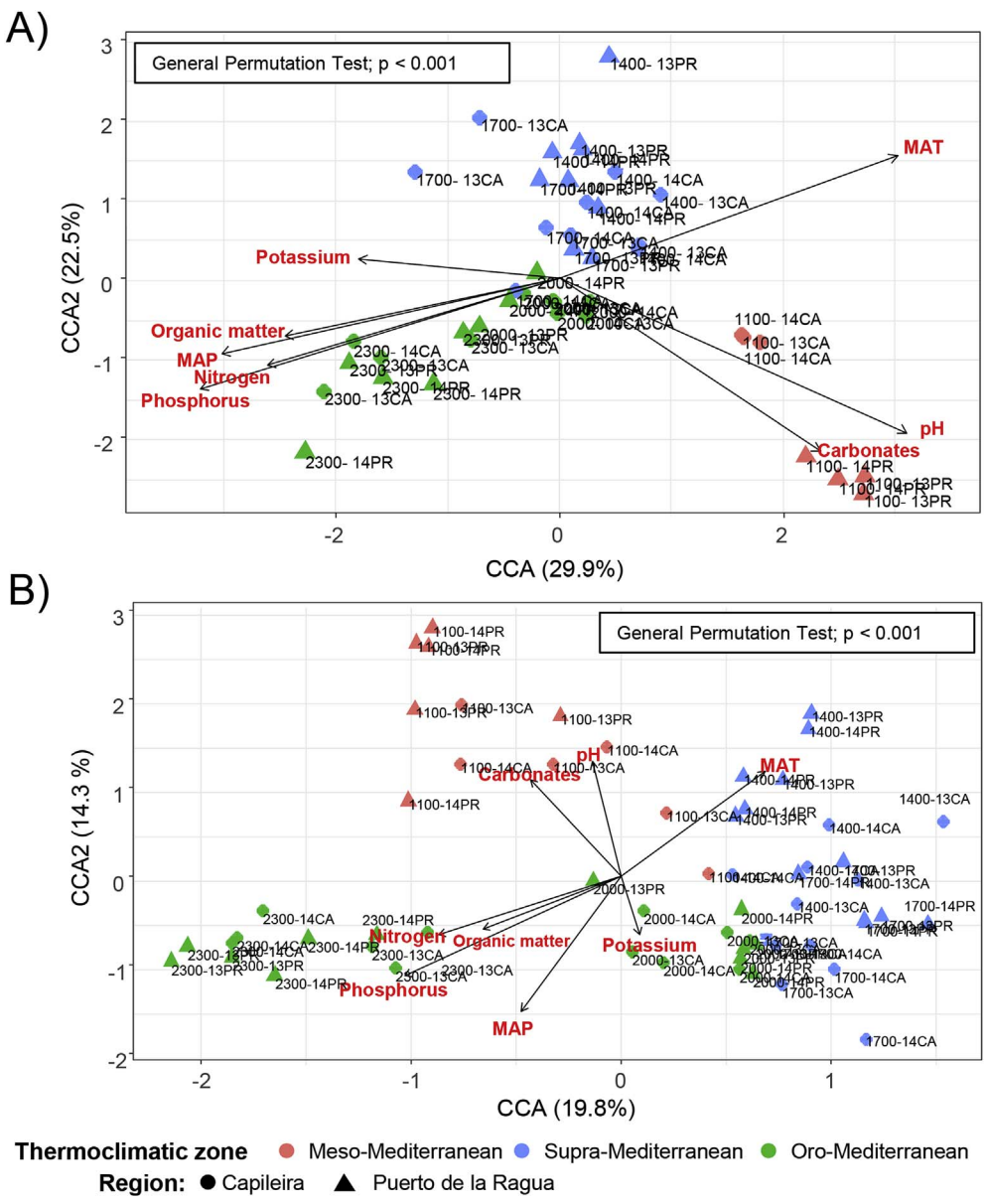

Fig. 3. Canonical Correspondence Analysis (CCA) of A) bacterial and B) fungal communities based on Bray Curtis distances among the different thermoclimatic zones in bulk soils. Data were constrained with the physicochemical characteristics of bulk soil and environmental parameters. Permutation tests confirmed the effect of the edaphic factors as drivers of the bacterial community ($p < .01$).

The taxonomic shifts observed along the different thermoclimatic zones and soil types suggest that specific phylotypes may exist which are particularly well adapted and prominent in each of these conditions. To investigate this possibility, the core bacteriome of bulk soil and the rhizosphere were determined at each thermoclimatic zone. In both the bulk and rhizosphere soils, the core-bacteriome with the largest number of OTUs was found in the Meso-Mediterranean zone (Fig. S5, Table S2). 2 OTUs were detected in the core bacteriomes of all three thermoclimatic zones and in both types of soils (HQ118340.1.1462 and DQ303296.1.1480; both *Bradyrhizobiaceae*) (Table S2). Interestingly, the relative abundance of the OTU HQ118340.1.1462 comprised from 1.3 to 7.1% of the total reads, suggesting that it is a cosmopolitan and possibly essential bacterium for the ecology of soils, independently of the presence of plant roots. 56 OTUs, mostly *Solirubrobacterales*, *Geodermatophilaceae*, *Bradyrhizobiaceae, Micromonosporaceae* and *Gaiellaceae*, were identified as members of the Meso-zone core bacteriome of both soil types. 18 OTUs identified as mostly *Mycobacteriaceae*, *Bradyrhizobiaceae* and *Hyphomicrobiaceae*, were shared by the core bacteriome of the Supra-zone of bulk and rhizosphere soils. 19 OTUs (mostly *Geodermatophilaceae*, *Micrococcaceae* and *Bradyrhizobiaceae*) were shared by the Oro-zone core bacteriome of both types of soils. From each core bacteriome and taking into account the differences in relative abundances of OTUs, potential bioindicators were identified which differentiate between the three thermoclimatic zones (Table S2). Very few core bacteriome OTUs could be identified which belonged exclusively to one thermoclimatic zone and had a certain relative abundance (> 0.14%). Nevertheless, two bioindicator OTUs exclusive for the Meso- Mediterranean zone, New. CleanUp.ReferenceOTU144588 (*Syntrophobacteraceae*) and New.ReferenceOTU730 (*Gaiellaceae*), could be identified in the bulk soil core. Other non-exclusive bioindicator OTUs could be identified for the Meso-Mediterranean zone such as 2 in bulk soil (KC554357.1.1538, *Rubrobacter*; and KC554358.1.1541, *Gaiellaceae*), 2 in the rhizosphere (DQ129295.1.1427, *Balneimonas*; and FJ946532.1.1290, *Burkholderiaceae*) and 2 more for both bulk soil and the rhizosphere (New.ReferenceOTU124, *Solirubrobacterales*; and JN178465.1.1494, *Balneimonas*). For the Supra-Mediterranean zone, 2 OTUs were identified in the bulk soil core (HQ597750.1.1457, *Solibacterales*; and New.ReferenceOTU708, *Solirubrobacterales*) and 2 OTUs in both the bulk soil and rhizosphere cores (New.ReferenceOTU467, *Solibacterales*; and FM209060.1.1379, *Mycobacterium*). For the Oro-zone, one bioindicator OTU could be identified in the bulk soil core (New.ReferenceOTU694,

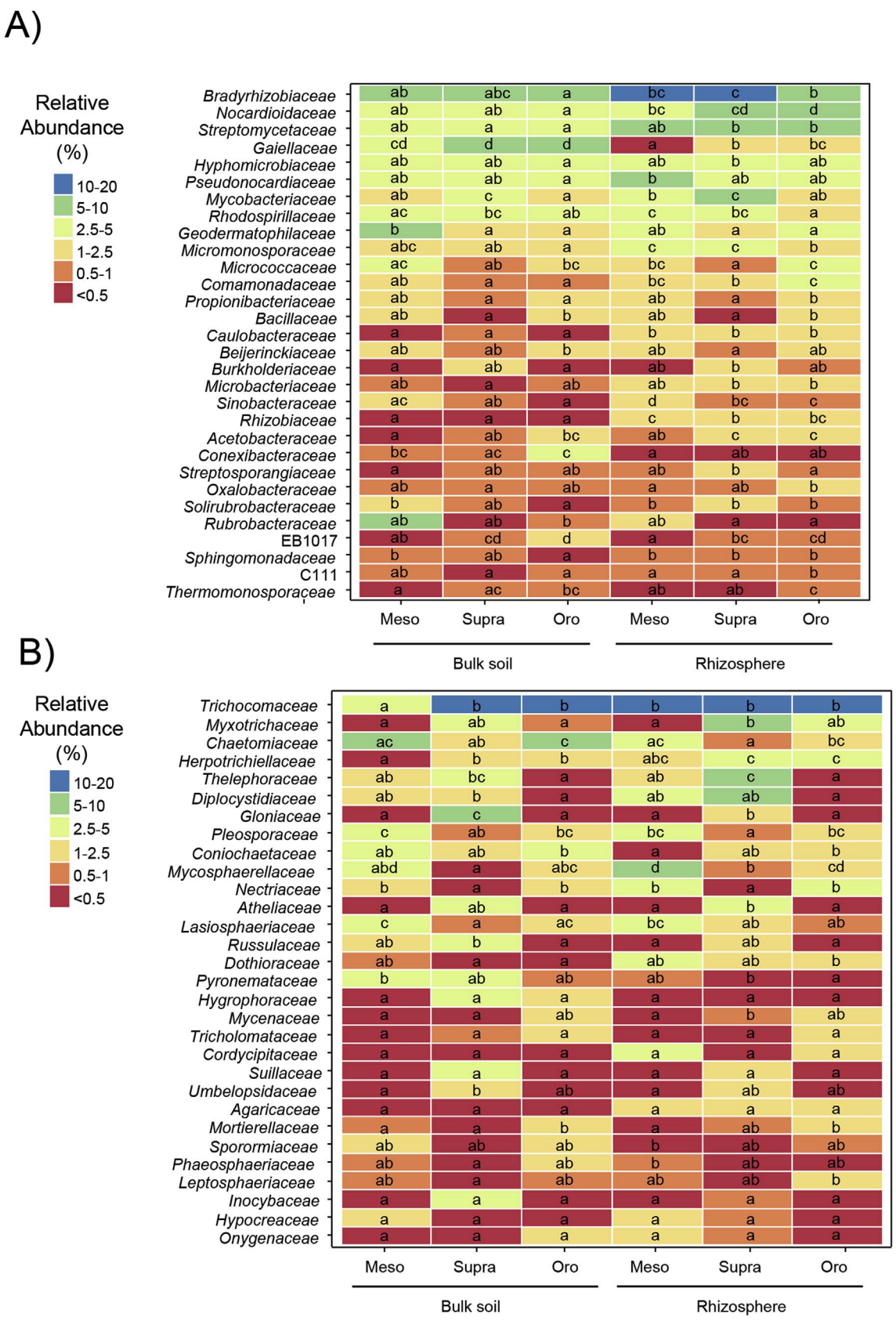

Fig. 4. Heatmap of the most abundant A) bacterial or B) fungal families (top 30) in the different soil types and thermoclimatic zones. To compare the relative abundance of each family among the three thermoclimatic zones in bulk soil and the rhizosphere the multcomp test was used. Different letters indicate significant differences ($p < .05$).

Bacillus) and 4 OTUs in the rhizosphere (AJ534679.1.1426, *Nakamurellaceae*; EF018802.1.1366 and EF019790.1.1319, both *Pseudonocardia*; and KC554446.1.1516, *Actinocorallia*). The fidelity and specificity of these OTUs for specific thermoclimatic zones was further corroborated by the indicator species analysis (IndVal) (Dufrene and Legendre, 1997). All the selected bioindicadors have high and significant IndVal scores.

3.3. Fungal community composition

The composition of fungal communities was determined by sequencing amplification products of the ITS2 intergenic region from each sample within the elevational transects. Base paired sequencing with MiSeq Illumina technology resulted in a total of 21,257,149 sequences. After filtering for quality and length, 14,952,588 high quality sequences with an average length of 298 bp were obtained. Just as with bacterial sequences, only OTUs with more than 0.005% relative abundance were considered for beta diversity calculations. The almost saturated rarefaction curves confirmed that the sampling effort was sufficient to cover the majority of the fungal diversity (Fig. S1B).

Similar to bacterial communities, the main deterministic factors for fungal community structure were the thermoclimatic zone (1st NMDS axis; Fig. 1B) and the type of soil (bulk or rhizosphere) (2nd NMDS axis). According to the NMDS plots and the PERMANOVA tests, neither the sampling year or transect location were strong deterministic factors for fungal communities (Fig. S2B). The thermoclimatic zone and soil type showed a reciprocal interaction (two-way PERMANOVA test; $p < .001$) and therefore, the effect of thermoclimatic zones was analyzed independently for either bulk soil or rhizosphere soil. The fungal communities collected at 1400 and 1700 m (both Supra- Mediterranean) or at 2000 and 2300 m (both Oro-Mediterranean) grouped separately in the NMDS plot (Fig. S2B), suggesting that, like with bacterial communities, the thermoclimatic effect was stronger than the altitudinal effect. The structure of the fungal communities at each of the three thermoclimatic zones was different independently of the type of soil (bulk or rhizosphere) analyzed (PERMANOVA test, $p < .001$). In addition, a progressive change of the fungal communities could be observed along the thermoclimatic gradient (Fig. 1B), with the fungal communities at the Meso- and Oro- Mediterranean zones showing the most similarity. Moreover, in both the bulk soil and the rhizosphere, the fungal communities at the three thermoclimatic zones remained different from each other (pairwise comparison PERMANOVA tests; $p < .05$). The heterogeneity of fungal communities in bulk soil, measured as Bray-Curtis pairwise intragroup distances, tended to decrease as the thermoclimatic zone descended (Fig. S3B). In the rhizosphere, the degree of dispersion of samples increased from the Meso-to Supra-Mediterranean zones, and then decreased again at the Oro- Mediterranean zone (Fig. S3B).

The alpha metrics of the fungal community increased clearly with ascending thermoclimatic zone in the rhizosphere although the differences in richness and evenness were not statistically significant (Fig. 2B). In the case of bulk soil, the Shannon index and evenness was lower in the Supra- Mediterranean zone than in the other two zones (Fig. 2B). Although the richness tended to increase progressively as the thermoclimatic zones ascended in a manner similar as observed in the rhizosphere, this trend was not statistically significant. In bulk soil, the richness of OTUs showed a negative linear correlation with carbonate content (Table 3). The Shannon's diversity index and the evenness of OTUs tended to increase as the concentration of assimilable phosphorus in soil was higher. However, the evenness of OTUs decreased linearly with the availability of assimilable potassium. In the case of rhizosphere fungal communities, richness and diversity (H′) showed significant linear correlations with MAT and MAP (Table 3), indicating that the environmental factors exercised a stronger effect driving the alpha diversity than in bulk soil.

The effect of edaphic and environmental properties driving the fungal communities although weak (15% of the total variation partitioning) was significant [general permutation test (999 replicates), $p < .001$] (Fig. 3B). The effect of the geographical location of the transects was less evident than in bacteria when the communities were constrained. The fungal community in the Meso-Mediterranean zone, like with bacteria, was structured strongly by pH and carbonate content. The community in the Supra-zone was driven partially by the MAT and the assimilable potassium content. In the Oro-community, the fungal community grouped as two independent clusters according to the altitude from which the samples had originated. The first cluster was mainly assembled by the amount of potassium and was more similar to the representatives of the Supra-region. The second group consisted of all the samples collected at 2300 m and were strongly regulated by the amount of organic matter, nitrogen and phosphorus. The MAP showed a weak effect in Supra- and Oro-communities (Fig. 3B).

The taxonomic structure of the fungal community was characterized by a clear numeric dominancy of the phyla *Ascomycota* and *Basidiomycota* independently of the thermoclimatic zone and the soil type (Fig. S4B). Four other phyla including *Chytridiomycota*, *Glomeromycota*, *Rozellomycota* and *Zygomycota* and a group of unassigned fungi were also present in the edaphic communities. At all thermoclimatic zones, the relative abundance of *Basidiomycota* was lower than *Ascomycota* although the relative abundance of this phylum increased significantly in the Supra-Mediterranean thermoclimatic zone. At the family level, *Trichocomaceae* was the dominant taxon in all the samples, except in the bulk soil of the Meso-Mediterranean zone where *Chaetomiaceae* was the dominant taxa (Fig. 4B). Some families showed similar trends along the thermoclimatic transects independently of the rhizosphere effect. The relative abundance of *Chaetomiaceae* and *Pleosporaceae* decreased at the intermediate Supra-Mediterranean thermoclimatic zone. *Thelephoraceae*, *Gloniaceae* and *Nectriaceae* showed a humpbacked trend while *Mortierellaceae* showed a constant increase with ascending thermoclimatic zone. Trends observed along the thermoclimatic transects which were more specific to soil type showed that in bulk soil, *Trichocomaceae* and *Herpotrichiellaceae* increased with ascending thermoclimatic zones whereas *Diplocystidiaceae* decreased. *Lasiosphaeriaceae* and *Umbelopsidaceae* showed their lowest relative abundance in the Supra-zone, while *Russulaceae* was the most abundant taxon in this thermoclimatic zone. On the other hand, in the rhizosphere soil *Coniochaetaceae*, *Mycenaceae* and *Sporormiaceae* tended to increase with ascending thermoclimatic zones, while *Pyronemataceae* tended to decrease. *Myxotrichaceae* and *Atheliaceae* showed a humpbacked trend in this soil, while *Mycosphaerellaceae* decreased in the Supra- Mediterranean zone.

Analyses of the core fungiomes of the three thermoclimatic zones indicated that independently of the type of soil (bulk or rhizosphere) the core fungiome of the Meso-Mediterranean zone harbored the largest number of OTUs (Fig. S5). The core fungiome was larger in the rhizosphere than the bulk soil. In the bulk soil, the numerically dominant OTU1666 (*Cryptococcus terreus*), was found in the core fungiome of all three thermoclimatic zones, while in the rhizosphere 2 OTUs were found in the core fungiomes of all three thermoclimatic zones (OTU189, *Pleosporales* fam *Incertae sedis*; and OTU370, *Penicillium*) (Table S3). Globally, the core fungiome consisted of a lower number of OTUs than the core bacteriomes (Fig. S5). A set of only 5 OTUs could be defined as potential bioindicators. For the Meso-Mediterranean thermoclimatic zone 4 bioindicator OTUs could be defined with 1 being found in bulk soil (OTU473, *Pleosporaceae* sp.), 2 in the rhizosphere (OTU300, *Paraphoma chrysanthemicola*; and OTU98, *Apiosordaria otanii*), and 1 in both bulk soil and rhizosphere cores (OTU42, *Mortierella antarctica*). In the Supra-zone no bioindicator OTU could be defined while in the Oro-Mediterranean zone, only one bioindicator OTU could be defined in the rhizosphere soil core (OTU162, *Holtermanniella takashimae*). The fidelity and specificity of these OTUs for specific thermoclimatic zones was further corroborated by IndVal in which all the selected bioindicadors

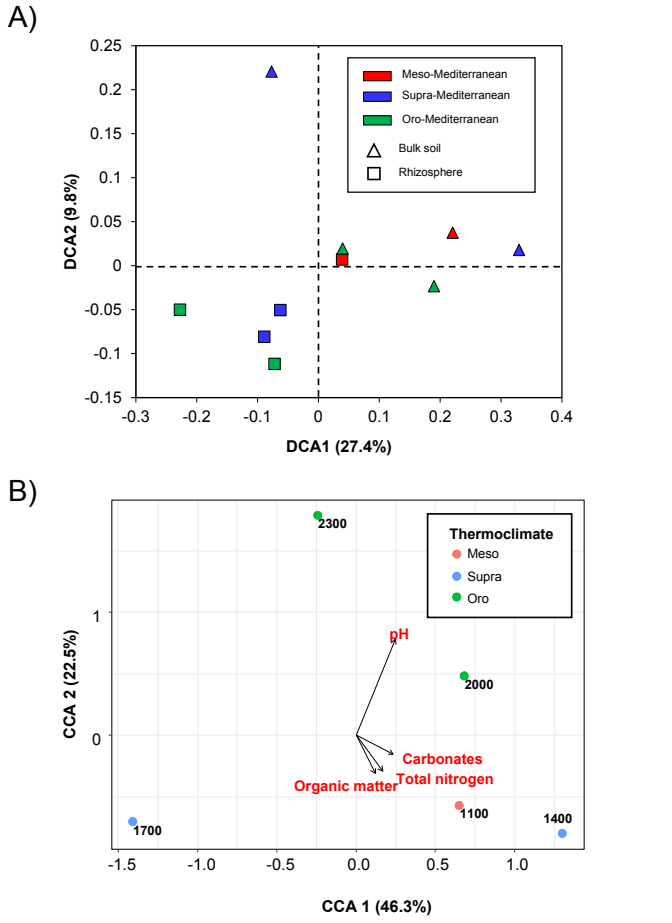

Fig. 5. A) Detrended correspondence analysis (DCA) of microbial functional gene community structure based on GeoChip 5.0 normalized signal intensity of the bulk and rhizosphere soils at the three thermoclimatic zones. B) Canonical Correspondence Analysis (CCA) of microbial functional gene community structure (Bray Curtis distances) among the different thermoclimatic zones in bulk soils. Data were constrained with physicochemical characteristics of bulk soil and environmental parameters. Permutation tests confirmed the effect the edaphic factors as drivers of the bacterial community ($p < .01$). Assimilable phosphorus, potassium, MAT and MAP constraints were aliased because they were co-linear.

have high and significant scores (Table S3).

3.4. Microbial functional gene diversity along a thermoclimatic gradient

To determine the microbial functional gene diversity along the thermoclimatic gradient, metagenomic DNA from a representative bulk soil and rhizosphere sample taken at each of the five altitudes in the region of Capileira in 2013 (a total of 10 samples) were analyzed using GeoChip microarrays (He et al., 2010). Samples were selected from a single spatial transect and year due to the weak effect that both explanatory factors showed on structuring microbial communities. A total of 63236 different functional genes were detected. These genes were mainly involved in seven functional categories: Metal homeostasis (26.5%), C cycling (16.2%), stress response (15.4%), virulence (14.7%), organic remediation (8.7%) and nitrogen metabolism (4.3%). Other genes involved in important ecological roles such as those related to sulfur and phosphorous metabolism, electron transfer and secondary metabolism were also detected but to a lower extent.

Detrended correspondence analysis (DCA) of the relative abundances of the functional genes (Fig. 5A) showed that altogether the soil type is the major factor driving functional microbial gene diversity (ANOSIM test, $p < .05$) unlike the thermoclimatic zone which did not exercise a statistically significant effect. According to Canonical Correspondence Analysis (CCA), environmental and edaphic properties were also not strong drivers of the microbial functional diversity in bulk soil (Fig. 5B). Despite that the richness, Shannon diversity (H′) and evenness of functional genes between bulk soil and rhizosphere were not significantly different, the richness and diversity tended to increase in rhizosphere soil (*t*-test; $p > .05$; Fig. S6). Similarly, the number of gene copies (measured as the total normalized signal intensity) tended to be higher in the rhizosphere although these differences were not significant (*t*-test; $p > .05$; Fig. S6).

The total abundance of genes associated with C degradation was larger in the rhizosphere than in the bulk soil (Fig. 6). Independently of the type of soil (bulk or rhizosphere), genes related to the degradation of camphor, a terpenoid synthesized by some plants, were numerically dominant (Fig. 6). The abundance of genes involved in the degradation of camphor was statistically similar among samples and are up to 10-fold more abundant than genes related to the degradation of complex polymers typically found in soils. Significant differences among samples were detected for genes related to the degradation of cellulose, chitin and starch (Fig. 6).

In the case of genes related to the nitrogen cycle, the most dominant functional genes resulted in the processes of ammonification (mostly glutamate dehydrogenase and phosphoglucosamine mutase genes), nitrification (ammonia monooxygenase and hydroxylamine oxidoreductase), assimilation of nitrogen (nitrate reductases) and denitrification (cytochrome cd1 nitrite reductase, nitrate reductase, nitrite reductase and nitrous-oxide reductase) (Fig. 6). As with C degradation, functional genes related to nitrogen metabolism were more abundant in the rhizosphere. Although no significant differences were observed for the major processes related to the N cycle among the three thermoclimatic zones, some tendencies could be observed. In bulk soil the relative abundance of nitrogen functional genes was higher in the Supra-zone, while in the rhizosphere the abundance increased with ascending thermoclimatic zone.

4. Discusion

4.1. Taxonomic response of microbial communities to thermoclimatic changes

The analysis of edaphic microbial community responses to environmental changes is useful to improve simulation models and mitigation strategies for climate changes (Siles and Margesin, 2017). The thermoclimatic gradients found in mountain ranges offer natural “laboratories” to study the effect of climate change on soil microbial communities (Bryant et al., 2008; Fierer et al., 2011; Sundqvist et al., 2013; Lin et al., 2015; Yasir et al., 2015; Lanzen et al., 2016; Siles et al., 2016; Siles and Margesin, 2017; among others). To date, most of the knowledge about the response of edaphic microorganisms to climate change arise from studies of bulk soils, neglecting the rhizosphere despite the key roles played by microorganisms which inhabit this niche for plant health and nutrition (Lakshmanan et al., 2014), the turnover of nutrients (Philippot et al., 2008) and for soil formation (Séguin et al., 2005; Drigo et al., 2008). In this study, the response of bacterial and fungal communities associated with bulk soil and the rhizosphere of wild thyme to thermoclimatic changes was investigated along altitudinal gradients in the Sierra Nevada Mountains (Spain).

This work confirms that the rhizosphere effect exercises a strong effect on the taxonomic assemblage and the diversity of edaphic microbial communities. In general, bacterial and especially fungal richness tended to be higher in the rhizosphere than in bulk soil, while the evenness was higher in the bulk soil. Similar observations have also been made with many other plant rhizospheres (Smalla et al., 2001; Yergeau et al., 2007; Inceoglu et al., 2011; Bakker et al., 2013; Berg et al., 2014; Edwards et al., 2015; Rehakova et al., 2015; Coleman-Derr et al., 2016; Baldrian, 2017; Kumar et al., 2017) and is mainly due to the flow of nutrients released by plants as root exudates to the

	Bulk soil			Rhizosphere		
	Meso	Supra	Oro	Meso	Supra	Oro
Carbon degradation						
Camphor	10223.1 a	9872.8 a	7542.5 a	10811 a	11394.7 a	10555.2 a
Cellulose	1207.4 ab	1326.7 ab	1130.4 a	1400.9 ab	1634.8 b	1610 a
Chitin	1444.2 a	1674.7 ab	1320.7a	1720.0 ab	1776.4 ab	1960.8 b
Cutin	1556.9 a	1800.4 a	1493.8 a	1977.8 a	2056.2 a	2284 a
Glyoxylate cycle	1307.3 a	1490.4 a	1330.9 a	1561.8 a	1563.7 a	1751.3 a
Hemicellulose	1671.9 a	1933.5 a	1542.5 a	2027.4 a	2040.4 a	2276.0 a
Inulin	1170.7 a	1348.9 a	1174.4 a	1776.6 a	1595.6 a	1913.5 a
Lactose	759.4 a	901.2 a	665 a	991.6 a	992.8 a	1077.7 a
Lignin	1187.5 a	1316.2 a	1099.4 a	1386.4 a	1433.8 a	1585.4 a
Pectin	1924.4 a	2148.4 a	1699.9 a	2188.3 a	2306.6 a	2466.6 a
Phospholipids	2353.2 a	2843.4 a	2058.6 a	2913.1 a	3035.8 a	3248.2 a
Protein	1165.2 a	1285.5 a	1083.4 a	1355.9 a	1436.6 a	1602.2 a
Starch	1580.2 a	1729.2 ab	1451.7 a	1874.2 b	1902.1 b	2097.4 b
Tannins	3435.7 a	3799.0 a	2797.4 a	3544.0 a	4121.1 a	4491.0 a
Terpenes	1750.7 a	2060.6 a	1640.3 a	2249.4 a	2182.9 a	2468.4 a
Vanillin/Lignin	1832.5 a	2005.0 a	1703.9 a	2065.8 a	2113.0 a	2380.2 a
Cyanide	1163.0 a	1428.5 a	1095.0 a	1365.6 a	1491.6 a	1609.1 a
Total Carbon degradation	35733.3	38964.4	30829.8	41209.8	43078.1	45377.0
Nitrogen cycle						
Ammonification	2068.8 a	2430.2 a	1894.8 a	2498.7 a	2461.1 a	2753.6 a
Anammox	667.2 a	1085.3 ab	775.7 ab	1056.7 ab	1103.6 ab	1436.3 b
Assimilation	2013.9 a	2355.4 a	1670.8 a	2266.7 a	2380.9 a	2466.4 a
Assimilatory N reduction	1249.0 a	1412.7 a	1160.6 a	1518.4 a	1504.5 a	1721.6 a
Denitrification	1516.1 a	1739.5 a	1375.6 a	1777.8 a	1855.5 a	2030.6 a
Dissimilatory N reduction	1115.7 a	1305.5 a	1028.7 a	1344.0 a	1435.0 a	1574.7
N Assimilation	961.3 a	1007.4 a	872.8 a	1072.0 a	1162.5 a	1330.0 a
Nitrification	1687.9 a	1961.5 a	1424.0 a	2027.0 a	2281.0 a	2509.2 a
Nitrogen fixation	1264.5 ab	1597.7 ab	1200.5 a	1560.2 ab	1692.1 ab	1856.7 b
Respiration	399.9 a	595.7 a	401.0 a	777.4 a	617.1 a	723.4 a
Total Nitrogen cycle	12944.3	15490.9	11804.5	15898.9	16493.3	18402.5

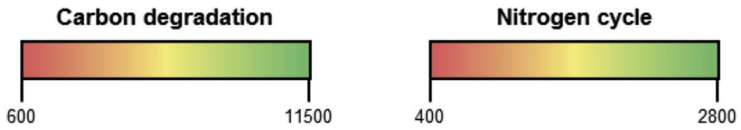

Fig. 6. Heatmap showing the means of the normalized signal intensities of genes related to carbon degradation and the nitrogen cycle detected at the Meso-, Supra- and Oro-Mediterranean zones in bulk soil and the rhizosphere of wild thyme. For each compound, values followed by different letters are significantly different (multcomp test; $p < .05$).

surrounding soil (Bais et al., 2006; Thion et al., 2016; Bulgarelli et al., 2013; Lebeis, 2015). With regard to the community composition, in the present study a proliferation of fast growing consumers of labile carbon from root exudates such as *Bradyrhizobiaceae* was observed in the rhizosphere while slow growing oligotrophic taxa such as *Streptomycetaceae* were more abundant in the bulk soil. These observations are in agreement with previous studies made with other plants (Fierer et al., 2007; Llado et al., 2017). Therefore, taking into account the

distinctiveness of the rhizosphere with regard to the surrounding bulk soil, the effects of other deterministic factors such as the thermoclimatic zone on microorganisms must be investigated separately for each type of soil.

In order to consider the potential temporal and spatial variability of microbial communities, samples were taken in two consecutive years and along two geographically distant altitudinal transects. Although microbial communities showed some variability between both years and transects, this variability was negligible in comparison with the effect derived from the type of soil (bulk or rhizosphere) and from the thermoclimatic zone. Nonetheless, the small variability observed due to the geographical location of each transect may be explained by the differences in some of the physicochemical soil properties such as the amount of carbonate and assimilable potassium. Similarly, seasonal variability of edaphic properties may take place and affect the microbial communities (Lazzaro et al., 2015; Lanzen et al., 2016). Therefore, to avoid this seasonal effect to a possible extent, sampling was performed within the same season each year.

In line with the initial hypothesis, bacterial and fungal diversity and community structure were found to be different along the three Mediterranean thermoclimatic zones studied. Furthermore, bulk soil and rhizosphere bacteria did not respond equally to the niche partitioning observed among the thermoclimatic zones. The response of microorganisms in bulk soil and the rhizosphere to the thermoclimatic zone were different with regard to the taxonomic composition of the communities and to the alpha diversity metrics. Since plant physiology and metabolism will also depend on the environmental conditions found in each thermoclimatic zone, the effect of thermoclimatic change along an altitude gradient may be magnified or buffered in the rhizosphere (Roy et al., 2013). In the current study, the differences in alpha diversity were more marked in the rhizosphere, while the effect on community composition was stronger in bulk soils.

The change of microbial community composition was not linear with altitude. The bacterial communities in the Meso- (lowest altitude) was closer to those found in the Oro- (highest altitudes) than to the Supra- Mediterranean zone (intermediate altitudes). The most different bacterial community was in the Meso-zone, with the differentiation between Supra- and Oro-zones being more marked for fungal communities. Previous studies have also shown an altitudinal effect structuring soil microbial communities (Bryant et al., 2008; Shen et al., 2013; Yuan et al., 2014; Jarvis et al., 2015; Siles and Margesin, 2017), where sometimes nonlinear trends were also observed (Singh et al., 2012, 2014; Lin et al., 2015). The strong niche partitioning observed along the thermoclimatic gradient is shown by the selection of specific bacterial taxa at each thermoclimatic zone independently of the rhizosphere effect. For example, two exospore forming bacteria such as *Thermomonosporaceae* and EB1017 resulted to be better adapted to higher thermoclimatic zones where the temperature is colder, water is more abundant and the pH is lower. *Bacillaceae*, an endospore forming bacterium preferred the Supra-Mediterranean zones, while *Mycobacteriaceae* became scarce in this intermediate zone. This niche partitioning was also shown by the high number of OTUs constituting the core microbiomes at each thermoclimatic zone.

The major drivers of microbial communities along the thermoclimatic zones were a combination of environmental and edaphic factors which changed concomitantly with the thermoclimatic zone. In the Meso- Mediterranean zone, the major drivers were pH and carbonate content, two interconnected parameters. Bacteria and fungi responded similarly to both parameters, confirming the importance of both of these variables for regulating the biosphere in this thermoclimatic zone. The microbial communities in the Supra-zone were structured mainly by the atmospheric temperature and to a lesser extent by the assimilable potassium. In the case of the Oro-zone, the major drivers were the availability of nutrients (nitrogen, phosphorous and organic matter content) and the availability of water measured as annual precipitation. In this study, MAP and MAT parameters showed a strong and significant linear correlation with altitude in both Capileira and Puerto de la Ragua transects. Both of these environmental parameters may exercise a strong effect on microbial communities due to the uniqueness of the Mediterranean macrobioclimate which is characterized by high temperatures and low rainfall during the summer months and low temperatures during the winter. Singh et al. (2014) similarly, observed that precipitation and temperature are deterministic factors for soil microbial communities. On the other hand, numerous studies of microbial diversity in biogeography and elevational gradients have shown that edaphic factors like pH (Bryant et al., 2008; Fierer et al., 2009; Lauber et al., 2009; Zinger et al., 2011; Shen et al., 2013; Rincon et al., 2015; Wang et al., 2015a; Zhang et al., 2015; Lanzen et al., 2016; Shen et al., 2016; Siles and Margesin, 2016) soil nutrient content such as the C/N content (Lanzen et al., 2016; Lin et al., 2015; Shen et al., 2015), dissolved organic carbon (Hofmann et al., 2016) or soil organic material (Zinger et al., 2011) are major drivers of microbial communities. However, it has been proposed that the structure of soil microbial communities along altitudinal gradients is ultimately governed by changes in the vegetation cover type (Singh et al., 2014), since the plant composition may affect soil pH, litter quantity and quality, soil nutrient content and soil moisture (Mitchell et al., 2010; Siles and Margesin, 2017). The ground cover in the Sierra Nevada Mountains is characterized mainly by shrubland (Meso-), pine forest and holm oak (Supra-) and meadow (Oro-). This shift of plant cover may help explain the linear, albeit not always significant, correlation with some edaphic properties. Namely, total nitrogen, oxidizable organic material and assimilable phosphorus correlated positively with altitude (albeit not always significantly), while pH correlated negatively. Similarly, an increase of C and other nutrients at higher altitudes due to the higher recalcitrance of coniferous litter (high C/N ratio and lignin content as well as low pH) has been reported in other mountain ranges such as the Alps (Siles and Margesin, 2017). This higher availability of nutrients may explain the increase of microbial (mainly fungal) species richness observed.

4.2. Response of microbial functional gene diversity to a thermoclimatic gradient

The assemblage and diversity of microbial functional genes was analyzed with the GeoChip 5 tool in order to determine whether a functional niche partitioning exists as a consequence of thermoclimatic changes. Contrary to the initial hypothesis and to previous reports (Siles and Margesin, 2017), the microbial functional gene diversity were not affected by thermoclimatic changes regardless of the rhizosphere effect. In addition, it was confirmed that in bulk soils, neither the environmental or edaphic properties showed a strong effect on the microbial functional diversity. Therefore, the taxonomic and functional microbial communities did not respond identically to thermoclimatic changes. This may be explained as a consequence of the functional redundancy in soil microbial communities (Andrén and Balandreau, 1999; Ding et al., 2015a, 2015b). On the other hand, a strong niche partitioning was observed for the type of soil (bulk or rhizosphere). The number and diversity of functional genes as well as the number of gene copies was larger in the rhizosphere of *T. zygis* than in bulk soil, indicating a higher predisposition of microorganisms inhabiting this niche to carry out more diverse and complex metabolic pathways. This larger genetic potential in the rhizosphere may be due to the greater number of plant-microorganism and microorganism-microorganism interactions on the one hand, and on the other to the greater quantity and diversity of nutrients which exist in this niche (Bais et al., 2006). Finally, it may also be stimulated in particular by the aromatic secondary metabolites produced by the wild thyme plant (Pascual et al., 2016). Although the differences among the three thermoclimatic zones were not significant, the richness and diversity of microbial functional genes in the rhizosphere tended to increase as the thermoclimatic zone ascends. This increase suggests that the plant stimulates or supports a larger

microbial gene pool at higher thermoclimatic zones.

Many of microorganisms inhabiting forest soils are characterized to be chemoorganotrophs which can use organic carbon sources such as decaying vegetal tissues containing recalcitrant and difficult to assimilate compounds (Baldrian, 2017; Llado et al., 2017). However, in this type of environment other alternative nutrient sources may be present such as nutrients released by plants as root exudates (Bais et al., 2006). In fact, the genes related to the degradation of camphor were those with higher normalized signal intensity in the microbial communities studied. Camphor is a terpenoid synthesized by some plants including thyme species (ElHadj Ali et al., 2010). Despite its antimicrobial activities (Sokovic et al., 2009), camphor can be catabolized by some microbial taxa. The dominance of genes related to camphor degradation was demonstrated not only in soil under the direct influence of wild thyme roots, but also in the bulk soil. Therefore, a potential diffusion of this organic compound to the surrounding soil may occur. Future experiments based on gas chromatography-mass spectrometry (GC-MS) will be needed to address this question.

Nitrogen is a limiting nutrient in many soils (Bulgarelli et al., 2013; Llado et al., 2017) and multiple studies have shown the importance of the nitrogen cycle in mountainous sites. In this study the nitrogen content of the soils increased with ascending thermoclimatic zone. A closer look at the functional diversity of nitrogen cycle genes detected with the GeoChip microarrays revealed higher gene copy numbers related to ammonification followed by nitrification, assimilation of nitrogen and denitrification. Therefore, microorganisms inhabiting the bulk soil and especially the rhizosphere are involved in key processes of the nitrogen cycle. Ammonification and nitrogen assimilation are immobilization processes which incorporate nitrogen into the microbial biomass and thereby make it unavailable for plant uptake while nitrification and denitrification are mineralization processes in which nitrogen is lost by microorganisms to the environment (Llado et al., 2017). Therefore, the present results, similarly to those observed by Cobo-Diaz et al. (2015), suggest that the edaphic microbial community has a genetic potential which favors nitrogen incorporation into the microbial biomass. The most notable differences between bulk and rhizosphere communities were observed in the Oro-zone, where the abundance of genes related to nitrogen fixation was higher in the rhizosphere. This implies a close interaction between the numerically dominant nitrogen-fixing bacteria like *Bradyrhizobiaceae* and the roots of wild thyme.

4.3. Microbial bioindicators of thermoclimatic changes

As is increasingly evident, global warming of the Earth's climate due to, among other factors, the greenhouse effect (Henderson et al., 2017) is causing increased concern that higher global temperatures will cause important consequences on ecological dynamics, including the balance between annual soil C inputs (photosynthesis) and losses (respiration) (Bradford, 2013). Currently, one of the greatest challenges is to develop powerful simulated models and mitigation strategies for climate change. This will require effective and meaningful bioindicators (Pulleman et al., 2012). Microbial bioindicators are typically used for monitoring alterations in the environment such as water or soil contamination (Sumampouw and Risjani, 2014). Good bioindicators must be found at high abundances, be wide-spread in a certain type of environment and have narrow and specific ecological demands and tolerances (Gerhardt, 2002) if they are to be useful for detecting climate change. Some examples are the result of efforts to define microbial indicator taxa that respond to soil warming (DeAngelis et al., 2015; Oliverio et al., 2017). Since the results of the present study show that thermoclimatic changes affect the edaphic microbial communities, useful bioindicators for climate change were sought by identifying representative bacterial and fungal OTUs from the core microbiomes of each of the three thermoclimatic zones studied. These criteria should ensure that the identified bioindicators are well-adapted to the specific ecological niches and environmental factors which characterize each thermoclimatic zone and not only to soil temperature. Altogether a set of 17 bacterial and 5 fungal bioindicators were selected, comprising several phylogenetic groups with different metabolism and physiology. The bacterial indicator OTUs defined here belong to taxa which in the study by Oliverio et al. (2017) were shown to have contrasting warm or cold responses thereby confirming that taxonomic identity does not necessarily predict the functional traits of the taxa (DeAngelis et al., 2015). Despite that the bioindicators were defined by taking into account the temporal (2 consecutive years) and the spatial variability scale (2 altitudinal transects in the Sierra Nevada Mountains), additional temporal (e.g. taking into account the seasonal variability) and spatial (e.g. in other mountain ranges with a Mediterranean climate) studies will be required to confirm their usefulness as bioindicators for thermoclimatic changes beyond the Sierra Nevada Mountains.

5. Conclusions

In this study thermoclimatic changes were shown to affect the taxonomic diversity and composition of edaphic bacterial and fungal communities. These effects were dependent on whether the microbial communities were associated with the rhizosphere of *T. zygis* or were found in bulk soils. This suggests that in contrast other elevational studies where climate factors had a less clear effect on microbial communities, the Sierra Nevada mountain range is an ideal location for studies regarding the effect of climate change on subsoil microbial communities. The study also shows that environmental and edaphic properties, some of which change linearly with altitude, are important drivers determining microbial community structures. On the other hand, the functional structure of microbial communities does not differ among the thermoclimatic zones, suggesting a functional redundancy along altitudinal gradients. Finally, a set of bioindicators were suggested for each thermoclimatic zone, which could be a starting point to monitor and to develop improved models for climate change in Mediterranean ecosystems.

Acknowledgements

This research was supported by project 590/2012 of the Network of National Parks (OAPN) within the Spanish Ministry of Agriculture, Food and the Environment. Additional financial support was obtained from the Spanish Ministry of Economy and Competitiveness (projects CSD2007-00005, BIO2010-17227), the Junta de Andalucía (project P12-BIO-772) and FEDER funds. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors are grateful to Joy vanNostrand and Delai He for help with GeoChip data analysis and to the authorities of the Sierra Nevada National Park in Spain for assistance and permits.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.12.013.

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